C1q-Deficiency Is Neuroprotective Against Hypoxic-Ischemic Brain Injury in Neonatal Mice

Vadim S. Ten MD, PhD; Sergei A. Sosunov, PhD; Sean P. Mazer, MD; Raymond I. Stark, MD; Casper Caspersen, PhD; Michael E. Sughrue, MD; Marina Botto, PhD; E. Sander Connolly Jr, MD; David J. Pinsky, MD

Background and Purpose—This study was undertaken to determine whether the initial component of the classical complement (C) activation pathway contributes to hypoxic-ischemic brain injury in neonatal mice.

Methods—Hypoxia-ischemia (HI) was produced in C1q−/− and wild-type (WT) neonatal mice. At 24 hours after HI, neonatal mouse reflex performance and cerebral infarct volume were assessed. Long-term outcomes were measured by water-maze performance and degree of cerebral atrophy at 7 to 8 weeks after HI. Activation of circulating neutrophils, and C1q, C3, and neutrophil deposition in brains were examined.

Results—C1q−/− mice were significantly protected against HI (mean±SE infarct volume in C1q−/− mice = 17.3±5.5% versus 53.6±6.8% in WT mice; P<0.0001) and exhibited significantly less neurofunctional deficit compared with WT mice. Immunostaining revealed significantly greater deposition of C3 (and C1q) as well as granulocytes in the infarcted brains in WT mice compared with C1q−/− animals. Activation of circulating leukocytes was significantly decreased in C1q−/− mice compared with WT mice, which correlated strongly (r=0.7) with cerebral infarct volumes.

Conclusions—Cerebral deposition of C1q and C3 after hypoxic-ischemic insult is associated with significantly greater neurologic damage in WT mice compared with C1q−/− mice, providing strong evidence that the classical C pathway contributes to the hypoxic-ischemic brain injury. Significantly decreased activation of circulating neutrophils associated with diminished local accumulation and attenuation of brain injury in C1q−/− mice suggests a potential cellular mechanism by which C1q mediates neurodegeneration in HI. (Stroke. 2005;36:2244-2250.)

Key Words: hypoxia • inflammation • ischemia • mice • neuroprotection

Rapidly evolving evidence indicates an active role of the complement (C) in cerebral ischemic injury in mature and immature animals.1–3 In human neonates, circulating C3 is depleted after birth asphyxia,4 and C-split products are increased in blood after fetal acidosis.5 Recently, it was demonstrated that pretreatment with cobra venom factor (CVF), a C-depleting agent, significantly reduced brain infarcts in p7 rats subjected to hypoxia-ischemia (HI).6 However, Lassiter et al reported previously that prehypoxic treatment with CVF did not result in cerebral protection in immature rats subjected to the same model of HI.7 Thus, conflicting results on pathogenic role of C activation in hypoxic-ischemic brain injury in immature rodents were obtained using the same species and model and the same neuroprotective strategy that prompted us to clarify whether C activation exacerbates hypoxic-ischemic brain injury in immature rodents.

Because CVF is not exclusively a C-specific inhibitor, we used C1q knockout (KO) mice and subjected them to a murine model of HI.8,9 The C1q was targeted because it has been shown that neurons actively synthesize this molecule in response to hypoxia.10 C1q mRNA has been reported to be dramatically upregulated in the brain after global ischemia11 and focal ischemic insult12 in rodents.

Materials and Methods

MURINE MODEL OF HIE

Newborn C57BL/6j mice of both genders were purchased from (Jackson Laboratories; Bar Harbor, Maine). C1q−/− mice were back-crossed into C57BL/6j for 10 generations.13 All research was conducted according to a protocol approved by the local animal care and use committee.

On p7, the hypoxic-ischemic insult was produced simultaneously in C1q−/− and wild-type (WT) pups by right carotid artery ligation followed by exposure to 8% oxygen/nitrogen for 20 minutes.8,9 The ambient temperature during hypoxia was monitored constantly at the level of 1.5 cm above the bottom of the chamber and was maintained at 37±0.5°C. Room temperature was maintained at 25 to 26°C. Carotid artery ligation without hypoxia (sham) produces neither brain damage14 nor functional impairments in rodents.15,16 Therefore, control animals consisted of age-, strain-, and genotype-matched naive mice.

Received February 6, 2005; final revision received May 26, 2005; accepted July 8, 2005.
From the Departments of Pediatrics (V.S.T., R.I.S., C.C.), Medicine (S.P.M.), and Neurosurgery (S.A.S., M.E.S., E.S.C.), Columbia University College of Physicians and Surgeons, New York, NY; Rheumatology Section (M.B.), Faculty of Medicine, Imperial College, London, UK; and Department of Medicine (D.J.P.), University of Michigan, Ann Arbor, MI.
Correspondence to Vadim S. Ten, MD, PhD, 3959 Broadway CHN 1201, New York, NY 10032. E-mail vt82@columbia.edu.
© 2005 American Heart Association, Inc.

Stroke is available at http://www.strokeaha.org DOI: 10.1161/01.STR.0000182237.20807.d0
Cerebral Blood Flow Measurement

In a separate cohort of WT and C1q<sup>−/−</sup> p8 mice, cerebral blood flow (CBF) changes during hypoxia and 10 minutes of reoxygenation were measured in both hemispheres using a laser Doppler flowmeter (Periflux 5000). In these mice at 2 hours after carotid artery ligation, the scalp was dissected under isoflurane anesthesia, and Doppler probes were attached to the skull (2 mm posterior and 2 mm lateral to the bregma) using fiberoptic extensions. Only local anesthesia (1% lidocaine) was used postoperatively. Mice then were placed into hypoxic chamber. Changes in CBF in response to hypoxia and reoxygenation were recorded and expressed as percentage of the prehypoxia level.

Neuropathological and Neurofunctional Definition of Injury

At 24 hours after HI, the sensorimotor mouse reflex (righting and geotaxis) performance was assessed as described previously.<sup>8</sup> Immediately after reflex assessment, mice were euthanized, and brains were stained with triphenyl-tetrazolium chloride (TTC) to assess infarct volume.<sup>8</sup> Late neurofunctional outcome was assessed in a separate cohort of adult WT and C1q<sup>−/−</sup> mice at 7 to 8 weeks after HI. Because of its superior sensitivity (to the rota-rod and open field tests), the water-maze test was chosen to assess the functional degree of neurodamage/neuroprotection and was performed according to a protocol.<sup>8,9</sup> After water-maze testing, mice were euthanized. Brains were coronally sectioned and Nissl stained for volumetric analysis of atrophy as described.<sup>8</sup>

Tissue Preparation and Immunohistochemistry

Brains harvested at 24 hours after HI were fixed in 4% paraformaldehyde for 12 hours at 4°C. Coronal sections (10 μm) were blocked and incubated with the anti-mouse C1q and C3 antibodies (Connex GmbH) at 4°C. Fluorochrome-conjugated secondary antibodies were used. Nissl staining was used as a nuclear counterstain. During confocal microscopy, the infarcted brain was identified by the regional loss of microtubule-associated protein-2 (MAP-2) immunopositivity. The level of immunostaining in cerebral tissue was assessed semiquantitatively by digital image analysis similar to the

Figure 1. Reflex performance (A and B) and cerebral infarct volume (C and D) at 24 hours after HI. E and F, Changes in the CBF during HI/reoxygenation. Data are mean±SEM. Study groups, genotype, and P values are indicated. HIE indicates hypoxic-ischemic encephalopathy.
Briefly, images of the area of interest (ipsilateral and contralateral hemisphere) were captured under identical fluorescence and magnification. Areas (in square pixels) of C1q- and C3-positive staining were measured using Image Pro Plus 4.5 software (Media Cybernetics). A total of 10 nonadjacent fields in 5 cerebral sections were analyzed from each mouse. Because it has been reported that maximal expression of C receptors was found at 24 hours after a cerebral focal ischemia, the 24-hour post-HI time point was chosen for C immunohistochemistry.

In a separate cohort of mice at 24 hours after HI, cerebral specimens were stained for deposition of Ly6C/G-positive cells, which are considered to be granulocytes. Briefly, specimens were cryosectioned as 10-μm slices. After blocking (0.3% H2O2 in PBS for 10 minutes at 4°C), specimens were incubated with biotin-conjugated anti-mouse Ly6C/G-antibodies (BD Biosciences). Ly6C/G-positive cells were identified by light microscopy as brown-colored cells on hematoxylin-stained background. To assess infarct volume in the same mice, 3 1-mm coronal sections from each mouse were stained with TTC.

In the same cohort of animals, 50 μL of blood was analyzed by flow cytometry for CD11b receptor expression on Ly6C/G-positive cells. Briefly, red blood cells were lysed by Fix and Lyse solution (BD Biosciences). The remaining cells were stained with anti-Ly6C/G and CD11b antibodies (BD Biosciences) for 20 minutes at 4°C. Neutrophils were defined using a distinct population gate: CD11bhi–Ly6C/Ghi. Fluorescence data were collected with a 4-color FACS Calibur (BD Biosciences) and analyzed using FlowJo software (Treestar). Because the flow cytometer had to be calibrated before each experiment, the actual value of CD11b-specific fluorescence for any given mouse was divided by the mean value of CD11b-specific fluorescence obtained in each precalibrated experiment to avoid a calibration-induced variability between experiments.

**Statistical Analysis**

Data expressed as means ± SEM. ANOVA and t test were used for comparison of brain infarct and atrophy, neurofunction, and neutrophil activation between groups. Linear regression analysis was performed to determine correlation between degree of cerebral injury and activation of circulating neutrophils. Data were considered significantly different if P < 0.05 between groups.

**Results**

**C1q−/− Neonatal Mice Are Protected Against HI**

C1q−/− pups exhibited significantly faster neonatal sensorimotor reflex performance compared with WT mice (Figure 1A and 1B). Cerebral infarct volume was significantly smaller in C1q−/− mice compared with WT mice (Figure 1C and 1D). Changes in CBF in response to hypoxia and reperfusion were similar in WT and C1q−/− mice (Figure 1E and 1F). Long-term outcome of neonatal HI in adult mice revealed that WT hypoxic-ischemic mice demonstrated significantly prolonged latency time to locate a submerged platform using peripheral navigational cues compared with WT naïve mice (Figure 2A). Although there was no statistical difference in latency time between C1q−/− hypoxic-ischemic mice and C1q−/− naïve mice (Figure 2A), navigational memory was significantly impaired in WT and C1q−/− hypoxic-ischemic animals compared with naïve counterparts. However, in C1q−/− hypoxic-ischemic mice, navigational memory was...
significantly better preserved compared with WT hypoxic-ischemic mice (Figure 2B). Residual volumes of ipsilateral hemisphere and hippocampus were significantly reduced in hypoxic-ischemic mice compared with those of naïve animals. When C1q−/− hypoxic-ischemic mice and WT hypoxic-ischemic animals were compared, ipsilateral hippocampal and hemispheric volumes were significantly better preserved in C1q−/− hypoxic-ischemic mice (Figure 2C and 2D).

C1q Deletion Decreases Neutrophil Activation and Infiltration
Flow cytometry of circulating neutrophils demonstrated significantly decreased expression of CD11b on Ly6C/G-positive cells in C1q−/− mice compared with WT mice after HI (Figure 3A). CD11b expression was similar in naïve and C1q−/− hypoxic-ischemic mice. In WT hypoxic-ischemic mice, CD11b expression was significantly increased compared with naïve WT animals (Figure 3A), which suggests that neutrophils were activated after HI. There was strong (r=0.7) correlation between degree of circulating neutrophils activation and cerebral infarct volume (Figure 3B). Microscopy of the infarcted brains obtained from the same hypoxic-ischemic mice demonstrated markedly increased neutrophilic infiltration in WT hypoxic-ischemic mice compared with similar brain regions in C1q−/− hypoxic-ischemic mice (Figure 3C through E). In a separate cohort of mice, granulocytes were counted in 10 high-powered (×40) fields in the cortex and striatum ipsilateral to the side of carotid artery ligation. Data included all hypoxic-ischemic mice from this cohort including one specimen from a C1q−/− mouse without a TTC-identifiable infarct. Quantification demonstrated significantly increased (P=0.04) presence of Ly6G/C-positive cells in WT mice (10.7±3.3; n=4) compared with C1q−/− counterparts (1.7±1.0; n=4).

Deposition of C3 Is Decreased in C1q−/− Hypoxic-Ischemic Mice
Immunohistochemistry of the brains from WT hypoxic-ischemic mice demonstrated deposition of C1q in the infarcted hemisphere but not in the unaffected hemisphere (Figure 4A), which was confirmed by semiquantitative analysis (Figure 4E). In C1q−/− hypoxic-ischemic mice, C1q positivity was not found either in the infarcted hemisphere or in the unaffected hemisphere (Figure 4B and 4E) as expected. Deposition of C3, the initial component of the terminal C cascade pathway, was present in the infarcted brain in WT and C1q−/− hypoxic-ischemic mice, suggesting that in C1q−/− mice, terminal C activation can be mediated by the alternative or MBL pathway (Figure 4C and 4D). However, in WT mice, C3 deposition in the infarcted brain was significantly increased compared with C1q−/− animals (Figure 4C through 4E). C3 deposition was localized in the vicinity of cells with remnant presence of the MAP 2 positivity (Figure 4C and 4D).
rather than in the necrotic core (complete loss of MAP-2 immunostaining) of the brain (data not shown).

Discussion
This study provides the first evidence that C1q-dependent activation of the C contributes to hypoxic-ischemic brain injury in neonatal mice because deletion of C1q gene confers significant and long-lasting neuroprotection. Significantly decreased deposition of C3 in the infarcted brain coupled with a lesser degree of neurodamage in C1q−/− mice compared with WT animals suggests that C3 activation plays an important role in the exacerbation of cerebral injury after HI.

To date, the most compelling data showing that C activation contributes to cerebral damage after neonatal HI have been reported by Cowell et al.6 These authors demonstrated neuroprotective effect of CVF-induced anti-C strategy in neonatal rats after HI. Data presented here are consonant with this result, although we used an anti-C strategy directed against the C1q-dependent pathway. In contrast to our findings and those of Cowell et al, Lassiter et al did not find evidence of anti-C–mediated neuroprotection against HI in rats.7 These conflicting data prompt further clarification.

There was a difference in the age of rats subjected to HI in the Cowell and Lassiter studies, being p7 (similar to the age of mice used in the presented study) in the former study and p21 in the latter. It is possible that in the Lassiter experiments, p21 rats had a more mature C system compared with p7 rats used by Cowell et al. The immature C system compared with a mature one may be depleted by CVF significantly faster because the immature C is already naturally depleted. C-split products produced in neonatal human serum by CVF-induced C activation (the mechanism of CVF-induced C depletion) was highly dependent on initial concentration of C components that were positively correlated with gestational age.21

In addition, the CVF alters not only C system but other systems that have been implicated in evolution of ischemic
by guest on April 12, 2017 http://stroke.ahajournals.org/ Downloaded from

Ten et al C1q Deletion Protects Against HI 2249

brain injury, such as platelet aggregation,22 and exhibits thrombin-like and proinflammatory activity.23 In this work, a single-gene KO neuroprotective strategy allowed us to highlight the specific C1q-mediated mechanism of hypoxic-ischemic brain injury.

There is a single report in which authors did not find significant neuroprotection in adult C1q−/− mice subjected to stroke.24 Although the model of brain injury used in this study (middle cerebral artery occlusion) is technically different from the HI model used in our experiments, the sequence of oxygen deprivation, followed by reperfusion, is common for both models. Therefore, one can speculate that age-related differences in C maturation may explain the discordant results. The p7 mice used in our study can be compared to 33- to 34-week-old premature infants.25 In premature neonates, the alternative pathway functionally26 and MBL pathway structurally and functionally are underdeveloped.27 The serum level of C9 is significantly decreased in neonatal compared with adult rats.28 Perhaps in neonatal mice with underdeveloped nonclassical pathways of C activation, genetic deletion of C1q results in inhibition of the terminal C cascade response to HI, which is sufficient to confer a neuroprotective effect. In mature animals, deletion of the C1q pathway alone may not suffice to inhibit terminal C cascade activation to a neuroprotective level because other nonclassical pathways of C activation are fully developed. This view is supported by our unpublished observation that demonstrated that C3−/− neonatal mice were protected against HI as well as C3−/− adult mice against middle cerebral artery occlusion stroke compared with C3+/- counterparts. Because C3 is the “meeting point” of all 3 pathways for C activation, genetic KO of C3 compared with isolated KO of C1q can theoretically provide more complete inhibition of the terminal C activation, which may offer a neuroprotection in immature and mature animals.

Another finding in this study is that the degree of activation of circulating neutrophils in response to HI strongly correlated with cerebral infarct volume and was significantly less of circulating neutrophils in response to HI strongly correlated with cerebral infarct volume and was significantly less in C1q−/− mice compared with WT animals. Because C1q is a potent mediator of neutrophil activation and degranulation,29 the absence of C1q-mediated stimuli for neutrophils in C1q−/− mice could explain decreased neutrophil activation and cerebral influx compared with WT mice. Neutrophil influx and degranulation in the injured brain after lateral fluid percussion head injury in rats was accompanied by accumulation of C3.30 Neutrophil accumulation in brain after injury is thought to be a contributing factor to ischemic damage.31 Our data suggest an important causal link between inflammatory cell recruitment and C1q-mediated exacerbation of HI-induced brain injury.

Histochemical detection of the deposition of C components on injured cells has been accepted as a marker of C cell interaction in neurodegenerative diseases32 and in traumatic or hypoxic-ischemic brain injury.2,6,7,30 In our study, deposition of C1q and C3 was found only in the injured cerebral tissue. Our data do not indicate the source (systemic or local) of these deposited C components in injured brain. Local constitutive and induced expression of C receptors (C3aR and C5aR) in postischemic cerebral tissue has been reported.33 In vitro, neurons are able to produce C1q C9 in response to hypoxia10 or inflammatory stimuli,34 which can culminate in C-dependent injury of neurons.17 Systemic depletion of C by CVF does not eliminate the presence of C3 or alter C9 deposition on injured neurons in immature hypoxic-ischemic rats.6 This suggests a local source of C deposition in post-HI brain.

In conclusion, our data are the first to demonstrate a C1q-mediated amplification of hypoxic-ischemic cerebral injury in immature mice. This result highlights the importance of the classical (C1q-dependent) C activation pathway in cerebral injury after HI insult and suggests that anti-C strategies against hypoxic-ischemic injury in immature brain can be targeted against C1q-mediated mechanisms of neurodegeneration.

Acknowledgments

This work was funded by the Bennett-Silverman Scholar Award (V.S.T.) and the National Institutes of Health (NS 40409 to E.S.C., NS 41460 and HL 59488 to D.J.P.). We gratefully acknowledge the helpful suggestions by Drs S. Vannucci and R. Polin during the performance of this work.

References

C1q-Deficiency Is Neuroprotective Against Hypoxic-Ischemic Brain Injury in Neonatal Mice
Vadim S. Ten, Sergei A. Sosunov, Sean P. Mazer, Raymond I. Stark, Casper Caspersen, Michael E. Sughrue, Marina Botto, E. Sander Connolly, Jr and David J. Pinsky

Stroke. 2005;36:2244-2250; originally published online September 22, 2005;
doi: 10.1161/01.STR.0000182237.20807.d0
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/36/10/2244

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/